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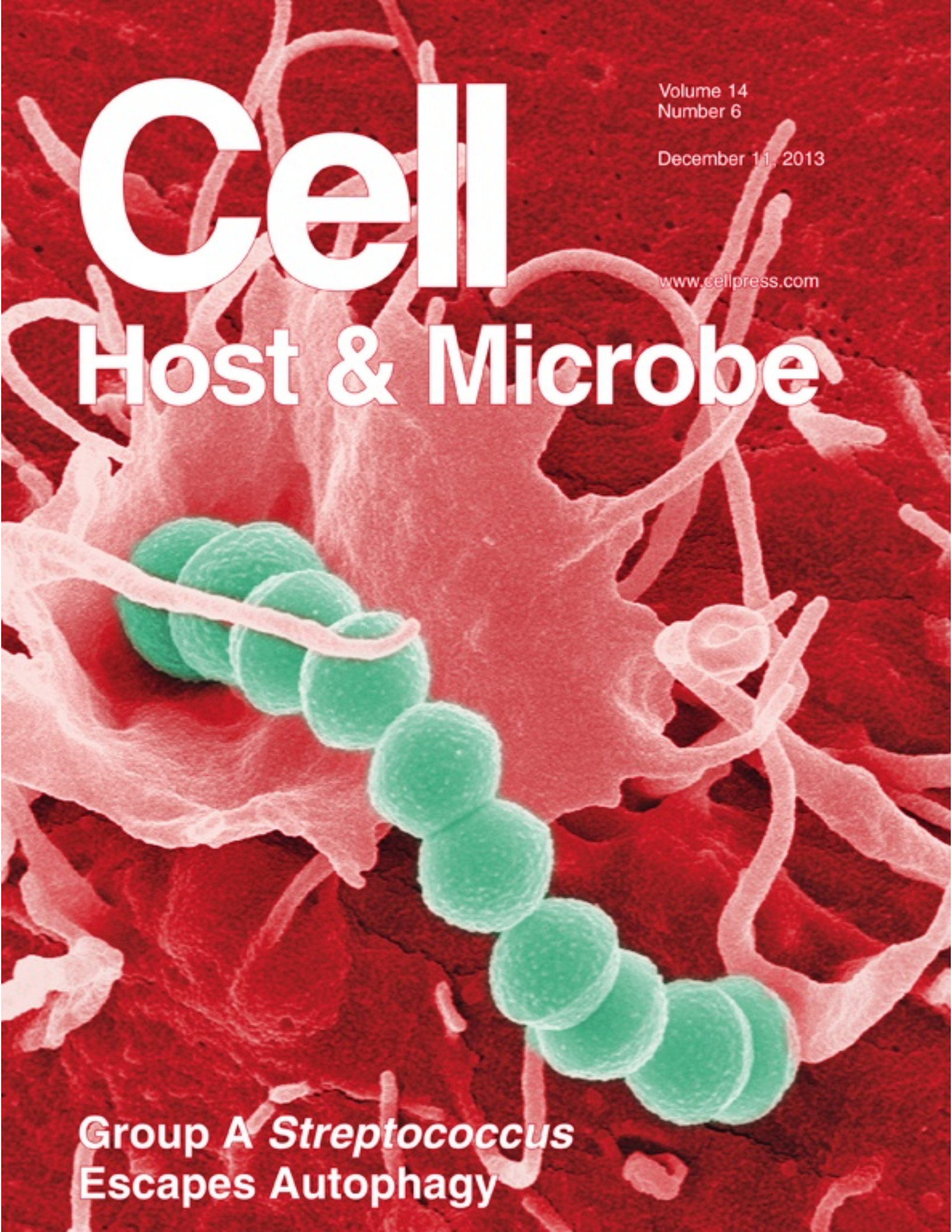

Group A *Streptococcus* Escapes Autophagy

Cell Host & Microbe
Short Article

The Globally Disseminated M1T1 Clone of Group A *Streptococcus* Evades Autophagy for Intracellular Replication

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SUMMARY

Autophagy is reported to be an important innate immune defense against the intracellular bacterial pathogen Group A *Streptococcus* (GAS). However, the GAS strains examined to date belong to serotypes infrequently associated with human disease. We find that the globally disseminated serotype M1T1 clone of GAS can evade autophagy and replicate efficiently in the cytosol of infected cells. Cytosolic M1T1 GAS (strain 5448), but not M6 GAS (strain JRS4), avoids ubiquitylation and recognition by the host autophagy marker LC3 and ubiquitin-LC3 adaptor proteins NDP52, p62, and NBR1. Expression of SpeB, a streptococcal cysteine protease, is critical for this process, as an isogenic M1T1 Δ*speB* mutant is targeted to autophagy and attenuated for intracellular replication. SpeB degrades p62, NDP52, and NBR1 in vitro and within the host cell cytosol. These results uncover a proteolytic mechanism utilized by GAS to escape the host autophagy pathway that may underpin the success of the M1T1 clone.

INTRODUCTION

Autophagy is a highly conserved cellular process that targets cytosolic components, including protein aggregates, damaged organelles, and intracellular bacteria, for lysosomal degradation, thus playing important roles in homeostasis and innate immunity (Deretic, 2010). Autophagy is an important cytosolic innate immune defense against bacterial infections (Huang and Brumell, 2009), and successful intracellular bacterial pathogens avoid autophagy by replicating in membrane-bound vacuoles or by camouflaging their surface with host- or bacterial-derived proteins (Dortet et al., 2011; Ogawa et al., 2005; Yoshikawa et al., 2009). Intracellular bacteria can be targeted to autophagy by a number of adaptor proteins that recognize polyubiquitylated bacteria in the cytosol or damaged bacteria-containing vacuoles (Kirkin et al., 2009; Thurston et al., 2009, 2012). These adaptor proteins, which include p62 (SQSTM1), NDP52 (CALCOCO2), NBR1, and optineurin, direct cargo to nascent LC3-positive phagophores and ultimately to degradation by the lysosomal pathway (Chong et al., 2012; Thurston et al., 2009; Wild et al., 2011; Zheng et al., 2009).

Group A *Streptococcus* (GAS) is an obligate human pathogen and the fourth most common bacterial cause of human mortality (Carapetis et al., 2005). The GAS disease burden ranges from superficial infections (pharyngitis, impetigo), to life-threatening invasive conditions (toxic shock, necrotizing fasciitis), to postinfectious immune disorders (rheumatic fever, glomerulonephritis) (Cole et al., 2011). A number of GAS strains are efficiently internalized into epithelial cells where they can be targeted to autophagy and cleared; however, these strains belong to serotypes M6 (Joubert et al., 2009; Nakagawa et al., 2004; Sakurai et al., 2010), M49 (Joubert et al., 2009), and M89 (Thurston et al., 2009), which are not representative of the prevalent serotypes associated with contemporary human disease epidemiology (Cole et al., 2011; Steer et al., 2009). Here, we show that the globally disseminated serotype M1T1 clone of GAS can replicate efficiently in the cytosol of infected cells through a process that involves proteolysis of the host proteins that target intracellular bacteria to autophagy.

RESULTS

M1T1 Strain 5448 Replicates within Epithelial Cells and Avoids Autophagy

While GAS has served as a model organism to unravel the complex molecular events that lead to antibacterial autophagy, the strains examined belong to serotypes infrequently associated with human disease. We therefore compared the intracellular survival of one such laboratory-adapted M6 strain (strain JRS4, hereafter $M6^{JRS4}$) (Nakagawa et al., 2004) with a recent clinical isolate of the globally disseminated serotype M1T1 clone (strain 5448, hereafter $M1T1^{5448}$) that has been the single leading cause of both pharyngitis and severe invasive GAS infections during the

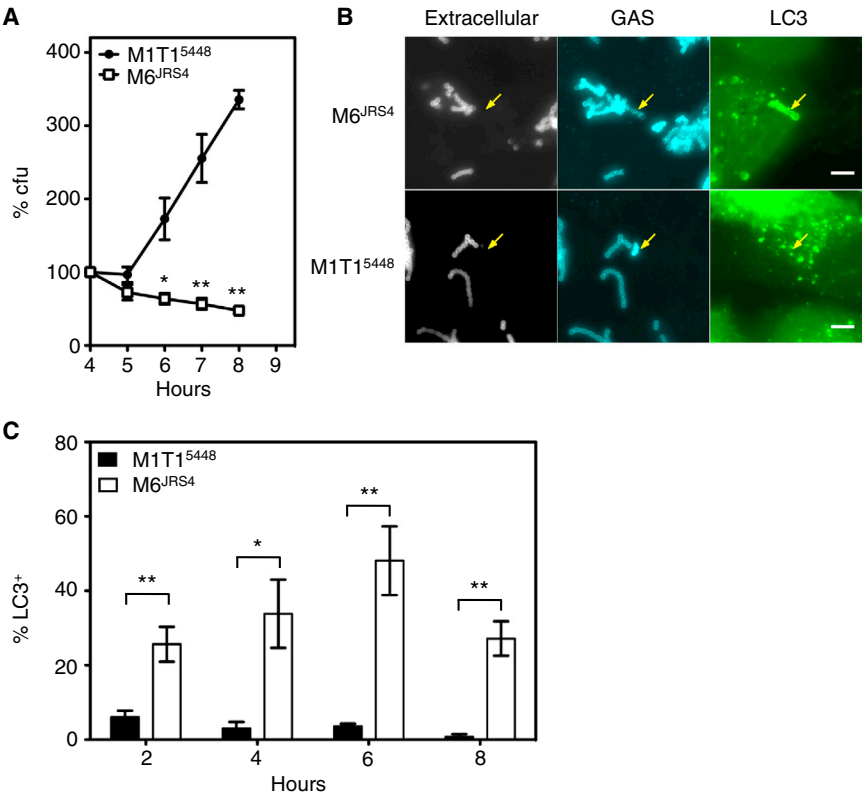

Figure 1. M1T1^{5448} Replicates within Epithelial Cells and Avoids Autophagy

(A) Ability of M1T1^{5448} and M6^{JRS4} GAS to survive following internalization into HEp-2 epithelial cells. Data are represented as mean ±SEM of three independent experiments. *$p < 0.05$; **$p < 0.01$; one-tailed paired t test.

(B) Association of intracellular M6^{JRS4} and M1T1^{5448} with GFP-LC3 at 4 hr postinfection. Arrows indicate intracellular M6^{JRS4} associated with GFP-LC3 and intracellular M1T1^{5448} devoid of GFP-LC3. Bar = 5 μm.

(C) Percentage of intracellular GAS contained within LC3-positive compartments. Values represent the mean ±SEM for three independent experiments. *$p < 0.05$; **$p < 0.01$; one-tailed unpaired t test.

last three decades. Intracellular viability of GAS following entry into human HEp-2 epithelial cells was monitored over time by measuring colony-forming units (cfu) (Figure 1A). Consistent with prior studies, the viability of the M6^{JRS4} strain decreased over time, as only 47% of cfu present at 4 hr postinfection remained at 8 hr postinfection. In contrast, recoverable cfu of the M1T1^{5448} strain tripled from 4 to 8 hr postinfection, revealing a capacity of this clinically important strain to not only survive, but also replicate, within epithelial cells.

To determine whether M1T1^{5448} intracellular replication reflected resistance to autophagy, we performed immunofluorescence microscopy to quantitate intracellular M1T1^{5448} or M6^{JRS4} GAS that colocalized with the autophagy marker GFP-LC3 (Figures 1B and 1C). M6^{JRS4} GAS were efficiently targeted to autophagy, with 48.1% ± 9.2% of intracellular M6^{JRS4} found in LC3-positive vacuoles at 6 hr postinfection. In contrast, only low numbers of M1T1^{5448} GAS were transiently associated with GFP-LC3 at 2 hr postinfection, and by 6 hr postinfection, the vast majority (96.4% ± 0.7%) were negative for GFP-LC3, demonstrating that the virulent human M1T1 isolate avoids targeting to autophagy to replicate within human epithelial cells.

M1T1^{5448} GAS Replicate in the Cytosol of Epithelial Cells

Autophagy primarily targets bacteria in the cytosol or in damaged membrane compartments, and bacterial pathogens such as *Salmonella* Typhimurium avoid autophagy by replicating within modified vacuoles (Birmingham et al., 2006). We therefore explored whether M1T1^{5448} avoids autophagy by replicating within an intact vacuole. To directly visualize intracellular M1T1^{5448} and M6^{JRS4} GAS, we performed transmission electron microscopy on GAS-infected HEp-2 cells at 6 hr postinfection (Figures 2A and 2B). The M1T1^{5448} strain was abundantly present in the cytosol of infected cells, whereas the M6^{JRS4} strain was contained within a membrane-bound compartment. To confirm that M1T1^{5448} was not associated with endosomal membranes, we performed immunofluorescence microscopy to quantitate the association of M1T1^{5448} with markers of early (EEA1) and late (Lamp1) endosomes (Figure 2C and Figure S1, available online). While transiently associated with endosomes at 2 hr postinfection, only 2.3% ± 0.7% of intracellular M1T1^{5448} were EEA1 or Lamp1 associated at 6 hr postinfection, suggesting that M1T1^{5448} escapes the endosomal pathway to replicate. We additionally examined the susceptibility of intracellular M1T1^{5448} to penicillin. Penicillin is an antibiotic that rapidly enters the cytosol, but not components of the endocytic pathway (Renard et al., 1987), a property that has been used to select for *Listeria monocytogenes* mutants that fail to escape the endocytic pathway (Camilli et al., 1989). Penicillin treatment abolished the intracellular replication of WT M1T1^{5448} with viability diminishing over the course of the experiment (Figure 2D). In contrast, penicillin treatment had minimal effect on the intracellular viability of M6^{JRS4} (Figure 2E). Taken together, these results suggest that the intracellular replication of M1T1^{5448} likely correlates to their successful entry into the cytosol of infected cells.

Intracellular M1T1^{5448} GAS Avoids Ubiquitylation and the Ubiquitin-LC3 Adaptor Proteins NDP52, p62, and NBR1

With few exceptions, ubiquitylation is a critical step in selective autophagy (Kirkin et al., 2009). LC3 is targeted to ubiquitylated bacteria via ubiquitin-LC3 adaptor proteins, which include p62, NDP52, NBR1, and optineurin (Chong et al., 2012; Deretic, 2010; Mostowy et al., 2011; Thurston et al., 2009; Wild et al., 2011). To determine whether M1T1^{5448} escapes autophagy by avoiding these pathways, we performed immunofluorescence microscopy on M1T1^{5448} and M6^{JRS4} infected cells to determine their respective colocalization with ubiquitylated proteins

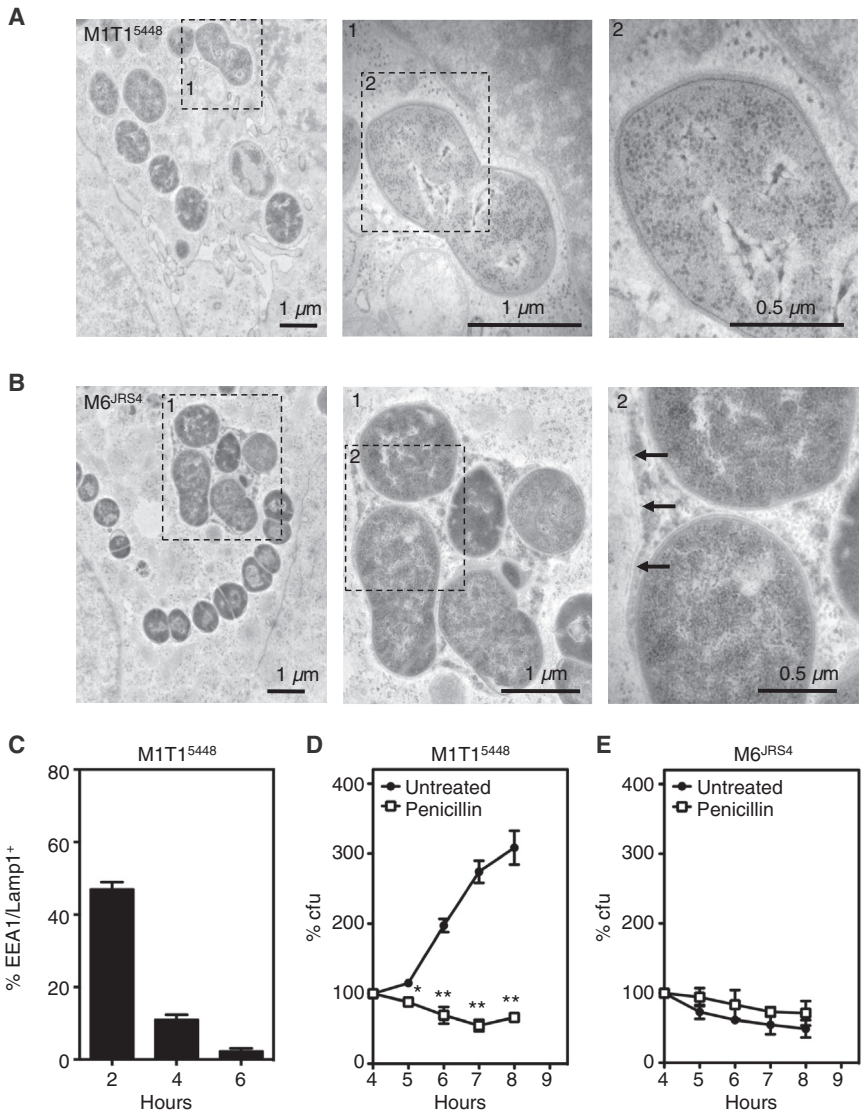

Figure 2. M1T1^{5448} Replicate Efficiently in the Cytosol of Epithelial Cells

(A and B) Transmission electron micrographs of HEp-2 cells infected with M1T1^{5448} (A) and M6^{JRS4} (B) GAS at 6 hr postinfection. The M6^{JRS4} GAS is entrapped within a membrane compartment with arrowheads indicating the membrane (zoomed micrograph on right); in contrast, the M1T1^{5448} GAS is entirely exposed to the cytosol.

(C) Percentage of intracellular M1T1^{5448} contained within EEA1- or Lamp1-positive compartments. Values represent the mean ±SEM for three technical replicates.

(D and E) Susceptibility of intracellular M1T1^{5448} (D) and M6^{JRS4} (E) GAS to penicillin. Values represent the mean ±SEM of three independent experiments. *$p < 0.05$; **$p < 0.01$; one-tailed paired t test. See also Figure S1.

(Figure 3A), NDP52 (Figure 3B), p62 (Figure 3C), and NBR1 (Figure 3D). Paralleling previous observations (Thurston et al., 2009), M6^{JRS4} GAS were found associated with NDP52, with the proportion of GFP-LC3-positive M6^{JRS4} GAS associated with NDP52 gradually increasing from 13.7% ± 3.0% at 2 hr postinfection to 33.3% ± 4.3% at 8 hr postinfection. However, the majority of GFP-LC3-positive M6^{JRS4} GAS were found associated with p62 (82.5% ± 3.7% to 96.3% ± 3.7%) and NBR1 (92.2% ± 4.0% to 98.4% ± 1.6%) at all time points examined (Table S1). In contrast, intracellular M1T1^{5448} GAS bacteria were not found in association with ubiquitylated proteins, NDP52, p62, or NBR1 (Figure 3), suggesting that intracellular M1T1^{5448} actively evades the autophagy pathways in epithelial cells.

SpeB Cysteine Protease Is Required for Efficient Intracellular Replication of M1T1^{5448} GAS

The M6^{JRS4} strain examined by Nakagawa et al. (2004) was previously shown to be defective in expression of the extracellular cysteine protease SpeB (Lyon et al., 2001) (Figure S2A), and is avirulent in a murine model of GAS infection (Figure S2B). Given that SpeB is a secreted and surface-associated protease (Hytönen et al., 2001) whose expression varies among GAS strains, we hypothesized that this virulence determinant may play a role in avoidance of the ubiquitylation system. Thus, we examined the ability of WT M1T1^{5448} and an isogenic Δ*speB* mutant to replicate within epithelial cells. Comparison of the genome sequences of M1T1^{5448} and M1T1^{5448} Δ*speB* revealed that these strains differ by only two SNPs (Figure S3). Compared to the WT parent strain, the M1T1^{5448} Δ*speB* mutant was attenuated for intracellular replication (Figure 4A), but not for replication in THY broth in vitro (Figure S3A). Furthermore, immunofluorescence microscopy revealed that, in comparison to wild-type (WT) M1T1^{5448}, targeting of the M1T1^{5448} Δ*speB* mutant to autophagy was much more efficient (Figures 4B and 4C), with 25.3% ± 3.9% intracellular M1T1^{5448} Δ*speB* associated with GFP-LC3 at 6 hr postinfection.

To further investigate the role of SpeB in resistance to autophagy, we engineered a M6^{JRS4} strain that expresses SpeB. In contrast to M6^{JRS4}, M6^{JRS4} + pSpeB replicated efficiently within HEp-2 cells (Figures 4D) and was only rarely associated with GFP-LC3 (Figure S2C). Similar results were also obtained with the SpeB-expressing strains M6^{MGAS10394} (Banks et al., 2004) and M12^{HKU16} (Tse et al., 2012), which replicated efficiently, compared to the naturally occurring SpeB-negative M4^{NS244} strain (McKay et al., 2004), which failed to replicate (Figure S2D).

SpeB Degrades Ubiquitin-LC3 Adaptor Proteins NDP52, p62, and NBR1

To determine whether SpeB conferred resistance to autophagy by its broad-spectrum cysteine protease activity, we purified SpeB from M1T1^{5448} (Figure S2E) and examined its ability to

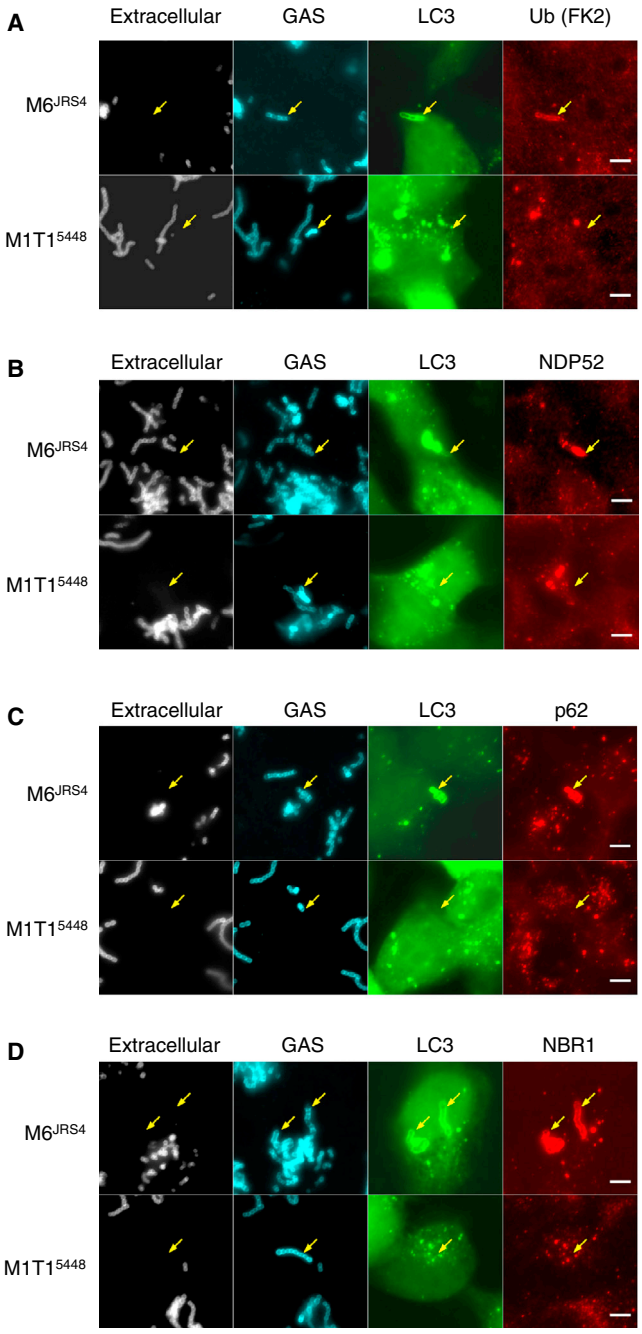

Figure 3. Intracellular M1T1^{5448} Avoids Ubiquitylation and the Ubiquitin-LC3 Adaptor Proteins NDP52, p62, and NBR1

(A–D) Association of M1T1^{5448} and M6^{JRS4} GAS with ubiquitylated proteins (A), NDP52 (B), p62 (C), and NBR1 (D). Quantitative data at 2, 4, 6, and 8 hr postinfection are provided in Table S1. Arrows indicate intracellular M6^{JRS4} associated with GFP-LC3 and adaptors, as well as intracellular M1T1^{5448} devoid of GFP-LC3 and adaptors at 4 hr postinfection. Bar = 5 μm. See also Table S1.

degrade components of the host ubiquitylation system. Purified SpeB efficiently degraded NDP52, p62, and NBR1 (Figure 4E), as well as ubiquitylated proteins (Figure S2F) from HEp-2 epithelial cell extracts. Similar results were obtained with bacterial culture supernatants from WT M1T1^{5448}, but not the isogenic Δ*speB* strain (Figure S2G). To confirm that these effects were specific to SpeB, we performed parallel experiments in the presence of the cysteine protease inhibitor E64 (Cole et al., 2007) (Figures 4E, S2F, and S2G). In a manner comparable to the mock-treated samples, the E64-treated, purified SpeB was deficient in the proteolytic activities described above.

We propose the ability of SpeB to act as a defense against host ubiquitylation components relies on its proteolytic activity within the environment of the host cytosol. To confirm that SpeB was enzymatically active in the host cytosol, we transfected HEp-2 cells with plasmids encoding either codon-optimized SpeB or a catalytically inactive C192S derivative and compared their effect on NDP52, p62, and NBR1 (Figures 4F and S2H–S2J). The plasmid encoding WT SpeB significantly reduced the number of NDP52, p62, and NBR1 puncta within transfected cells when compared to the catalytically inactive C192S mutant, demonstrating that WT SpeB is enzymatically active within the host cell cytosol. Taken together, these results demonstrate that GAS SpeB protease is necessary and sufficient to degrade ubiquitylation components within the host cytosol that normally serve to direct the bacterium to autophagy.

DISCUSSION

Autophagy primarily targets bacteria in the cytosol or in damaged membrane vacuoles. Some intracellular bacterial pathogens, such as *Salmonella* Typhimurium (Birmingham et al., 2006) and *Staphylococcus aureus* (Schnaith et al., 2007), avoid autophagy by replicating within modified endocytic or autophagic compartments. Other bacterial pathogens, such as *Shigella flexneri* and *Listeria monocytogenes*, escape the endocytic pathway and replicate in the cytosol of infected cells, avoiding ubiquitylation by camouflaging their surface with bacterial or host-derived proteins (Dortet et al., 2011; Ogawa et al., 2005; Yoshikawa et al., 2009). Here we provide several lines of evidence that GAS employ a proteolytic mechanism to evade autophagy and replicate in the cytosol of infected cells: (1) genetic deletion of the gene encoding SpeB significantly reduced the intracellular replication of M1T1^{5448}; (2) genetic deletion of the gene encoding SpeB from the M1T1^{5448} strain significantly increased the frequency of the recruitment of LC3 to the surface of the intracellular bacteria; (3) expression of SpeB by M6^{JRS4} promoted intracellular replication and abolished recruitment of LC3 to the bacterial surface; (4) purified SpeB degrades the ubiquitin-LC3 adaptor proteins p62, NDP52, and NBR1; (5) ectopic expression of codon-optimized SpeB in HEp-2 cells reduced the amounts of p62, NDP52, and NBR1; and (6) WT SpeB-expressing strains replicate efficiently in epithelial cells while naturally occurring SpeB-defective strains fail to replicate. Therefore, we propose that production of a bacterial protease that degrades the host proteins responsible for targeting bacteria to autophagy, in addition to proteolytic degradation of ubiquitylated bacterial surface proteins, constitutes a previously unrecognized mechanism employed by a bacterial pathogen to evade autophagy.

GAS is a highly successful pathogen of its human host, causing a wide array of superficial and invasive diseases. The transition from superficial to invasive disease involves a genetic

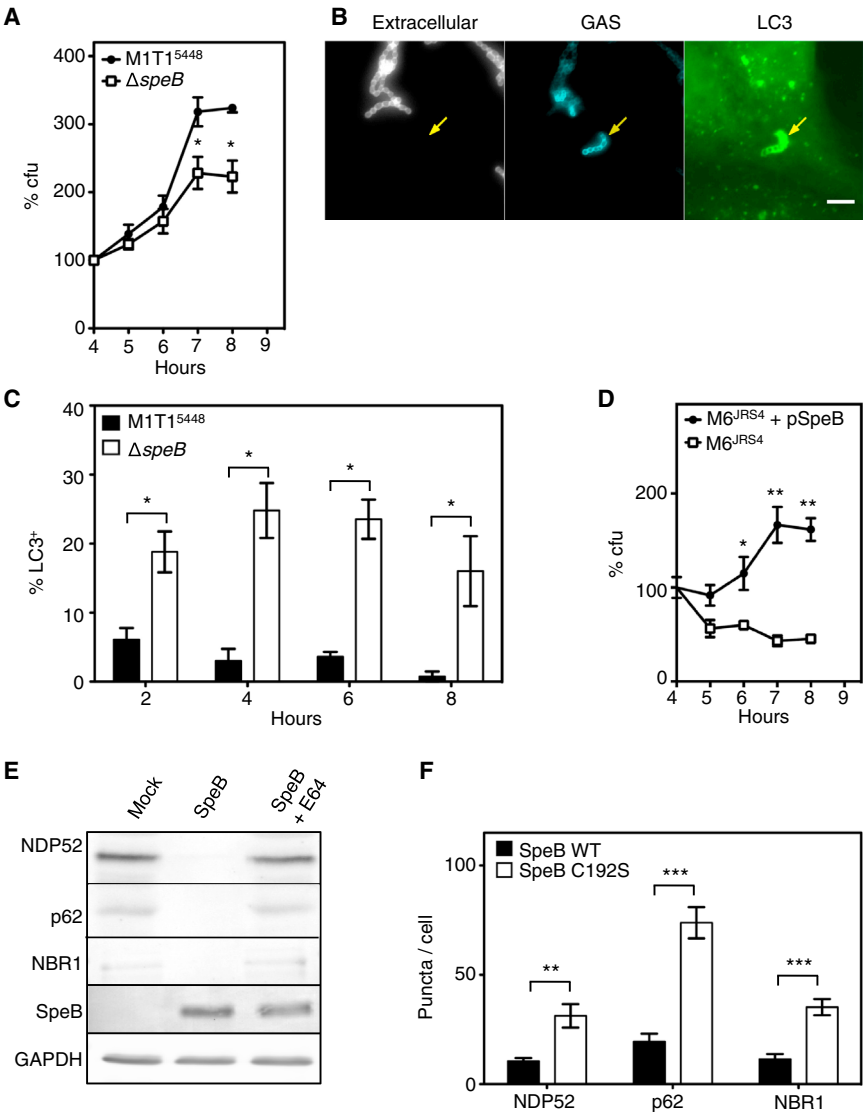

Figure 4. SpeB Cysteine Protease Degrades Ubiquitin-LC3 Adaptor Proteins and Is Required for Efficient Intracellular Replication of GAS

(A) Ability of M1T1^{5448} Δ*speB* to replicate within HEp-2 epithelial cells. Data are represented as mean ±SEM of three independent experiments. *p < 0.05; **p < 0.01; one-tailed paired t test.

(B) Association of M1T1^{5448} Δ*speB* with GFP-LC3 at 4 hr postinfection. Arrows indicate intracellular M1T1^{5448} Δ*speB* mutant associated with GFP-LC3. Bar = 5 μm.

(C) Percentage of intracellular GAS contained within LC3-positive compartments. Values represent the mean ±SEM of three independent experiments. *p < 0.05; one-tailed paired t test. The M1T1^{5448} data were taken from Figure 1C and are included for comparison.

(D) Intracellular replication of the SpeB-expressing M6^{JRS4} + pSpeB strain compared with the SpeB-negative M6^{JRS4} strain. Data are represented as mean ±SEM of three technical replicates. *p < 0.05; **p < 0.01; one-tailed unpaired t test.

(E) Western immunoblot showing that purified SpeB cysteine protease degrades NDP52, p62, and NBR1 in HEp-2 cell lysates.

(F) Ectopically expressed SpeB degrades cytosolic NDP52, p62, and NBR1. Bars represent the number of puncta ±SEM in at least 15 transfected cells and are representative of two experimental replicates. **p < 0.01; ***p < 0.001; one-tailed paired t test. Representative images used for the quantitation are shown in Figures S2H–S2J. See also Figure S2.

switch that abolishes SpeB expression while concomitantly increasing expression of numerous virulence factors required for growth in deeper tissues (Cole et al., 2011). As such, there is a strong correlation between SpeB expression and superficial disease (Cole et al., 2011; Ikebe et al., 2010). SpeB is a broad-spectrum cysteine protease required for virulence (Cole et al., 2006) that has been shown to degrade a number of immunologically important human proteins, including immunoglobulins, chemokines, proinflammatory cytokines, and cathelicidin, and to proteolytically modify GAS surface proteins such as M protein, which is important for host colonization (Johansson et al., 2008; Nelson et al., 2011). Additionally, SpeB has been shown to bind to a variety of host proteins, including laminin (Hytönen et al., 2001) and integrins (Stockbauer et al., 1999), which may have undescribed influences on host cell differentiation and signaling. Together, these properties suggest that SpeB expression is required by GAS for multiple aspects of epithelial colonization, which includes resistance to autophagy and other innate defenses.

In addition to SpeB expression, there is also a strong correlation of certain GAS serotypes with superficial disease in developed countries; M1T1 and M12 strains are the most prevalent in published epidemiological studies (Shulman et al., 2009). In contrast, M6 (Joubert et al., 2009; Nakagawa et al., 2004; Sakurai et al., 2010), M49 (Joubert et al., 2009), and M89 (Thurston et al., 2009), which have been used in prior GAS autophagy studies, are much less frequently associated with human disease. It is likely that M1T1 and other prevalent serotypes possess other genetic traits that enhance their success at colonizing epithelial surfaces (Maamary et al., 2012). In agreement with this is our observation that the M1T1^{5448} Δ*speB* strain was not as attenuated as M6^{JRS4}, suggesting that M1T1^{5448} has other intrinsic mechanisms of resistance to ubiquitylation and autophagy. Such mechanisms may include additional surface-bound proteins that mask the bacterial surface or subvert autophagy or, alternatively, the absence of bacterial targets for ubiquitylation. In addition, our data also illustrate the importance of investigating virulent strains of bacterial pathogens rather than relying exclusively on lab-adapted strains such as M6^{JRS4}, as lab-adapted strains may not provide a true representation of the phenotype of a bacterial pathogen in a particular disease model.

In conclusion, we report here a previously unrecognized strategy employed by an intracellular bacterial pathogen to manipulate the host autophagy pathway. By production of a protease that degrades host proteins that target bacteria to autophagy, a strain of the globally disseminated M1T1 GAS serotype can evade autophagy and replicate efficiently in the cytosol of infected epithelial cells. We propose that SpeB is required by GAS strains to establish successful colonization of epithelial tissues, thereby allowing such strains to escape an important component of host innate defense.

EXPERIMENTAL PROCEDURES

Bacterial Growth

GAS strains $M6^{JRS4}$ (Nakagawa et al., 2004), $M1T1^{5448}$ (Kansal et al., 2003), $M1T1^{5448}\Delta speB$ (Kansal et al., 2003), $M6^{MGAS10394}$ (Banks et al., 2004), $M12^{HKU16}$ (Tse et al., 2012), and $M4^{NS244}$ (McKay et al., 2004) were grown in Todd-Hewitt medium with 0.2% yeast extract (THY) at 37°C. Strain $M6^{JRS4}$ + pSpeB was constructed by introducing plasmid pSpeB (Korotkova et al., 2012) into $M6^{JRS4}$ by electroporation.

Cell Culture and Transfections

Cell lines were cultured at 37°C in 5% CO_2. HEp-2 cells were originally obtained from ATCC and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco). Plasmid DNA was introduced into HEp-2 cells using Attractene reagent (QIAGEN).

GAS Intracellular Growth Assay

For GAS infection, early stationary-phase bacteria (OD_{600} = 1.0–1.2) were harvested and washed once with Dulbecco's phosphate buffered saline (DPBS [pH 7.4]). The bacteria were diluted into 1% FBS/DMEM and added to confluent cell culture monolayers at a multiplicity of infection equal to 0.8 (eight bacteria for every ten cells). After 2 hr, cells were washed once with 10% FBS/DMEM and then incubated in 10% FBS/DMEM with 100 μg/ml gentamicin to kill extracellular bacteria. Where indicated, 100 U/ml penicillin G was also included. After an appropriate incubation time, infected cells were washed twice with PBS, treated with 200 μl trypsin, and lysed by the addition of 0.025% Triton X-100. Cell lysates were serially diluted and plated on THY agar for bacterial enumeration.

Construction of mRFP-LC3

A plasmid encoding LC3 fused to the C terminus of mRFP was constructed by inverse-PCR using primers delEGFP-S (5′-CGAGCTGTACAAGTCCGGACTC AGATCTC-3′) and delEGFP-A (5′-GCATAGATCTCTTGCTCACCATGGTGGC GAC-3′) and a plasmid encoding mRFP-GFP-LC3 (Kimura et al., 2007) as template DNA. The resulting PCR amplicon was digested with BglII and ligated with T4 DNA ligase to create pmRFP-LC3.

Antibodies

Primary antibodies used were rabbit anti-group A polysaccharide (PAB13831, Abnova), rabbit anti-GAPDH (2275-PC-100, R&D Systems), rabbit anti-SpeB (PBI222, Toxin Technology), mouse anti-EEA1 (610457, BD Transduction Laboratories), mouse anti-Lamp1 (CD107a; 555798, BD PharMingen), mouse anti-p62 (TA502239, OriGene), mouse anti-NDP52 (TA501971, OriGene), mouse anti-NBR1 (B01P, Abnova), mouse anti-ubiquitylated proteins (FK2, Millipore), mouse anti-ubiquitin (P4D1, Cell Signaling Technology), and rabbit anti-myc (Novus Biologicals). Secondary antibodies used for immunofluorescence microscopy were Alexa Fluor-conjugated goat anti-mouse or goat anti-rabbit antibodies (Invitrogen). Secondary antibodies used for western immunoblots were IRDye680LT-conjugated goat anti-rabbit or IRDye800CW-conjugated goat anti-mouse (LI-COR).

Immunofluorescence Microscopy

HEp-2 cells cultured on glass coverslips were infected with GAS strains as described above. At the indicated times postinfection, coverslips were washed four times with 500 μl DPBS and fixed in 4% paraformaldehyde (PFA). The fixed cells were blocked in DPBS containing 2% BSA and 0.02% sodium azide. Extracellular bacteria were stained with anti-group A polysaccharide antiserum. Coverslips were again fixed in 4% PFA, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked in DPBS containing 2% BSA, 0.1% Triton X-100, and 0.02% sodium azide (PBS-BT). Coverslips were stained with primary antibodies and AlexaFluor-conjugated secondary antibodies (Invitrogen) in PBS-BT, mounted onto glass slides, and imaged on a Personal Deltavision inverted microscope (Applied Precision). Maximum projection of acquired images was performed using the ImageJ Fiji software package (http://fiji.sc/wiki/index.php/Fiji).

SpeB Activity Assay

HEp-2 cell lysates were prepared by lysing confluent monolayers in T75 flasks (~8×10^6 cells) in 150 μl DPBS containing 1% Triton X-100, 0.1% SDS, and EDTA-free complete protease inhibitor (Roche). Lysates were passed ten times through a 27G needle and insoluble material removed by centrifuging at 14,000 × *g* for 15 min. The resulting supernatant was dialyzed extensively against DPBS before use.

SpeB was purified from stationary-phase $M1T1^{5448}$ culture supernatants using a modification of the protocol described by Berge and Björck (1995). Briefly, SpeB was precipitated from a 500 ml 24 hr culture supernatant of $M1T1^{5448}$ with 80% w/v ammonium sulfate. The resuspended precipitate was dialyzed against 5 mM MES (pH 6.0) and purified by affinity chromatography on SP-sephadex column eluted with a 5–250 mM MES (pH 6.0) stepwise gradient and dialyzed extensively against PBS.

Susceptibility of ubiquitin components to SpeB was measured using a modification of the method described by Collin and Olsén (2000). Filtered 24 hr culture supernatants were incubated for 30 min at 40°C with an equal volume of activation buffer (20 mM DTT in DPBS). Purified SpeB (644 nM final concentration following addition of HEp-2 cell lysate) was activated in activation buffer for 30 min at 40°C. After activation, an equal volume of HEp-2 cell lysate was added, and 10 μl was removed for a loading control. Reactions were incubated for 120 min at 37°C and stopped by the addition of 1× SDS-PAGE loading buffer and 100 mM DTT followed by heating at 95°C for 7 min. Samples were separated by SDS-PAGE, transferred to Immobilon-FL PVDF membranes, and probed with specific antibodies. Blots were visualized using IRdye680LT and IRdye800CW-conjugated secondary antibodies (LI-COR) and scanned on an Odyssey infrared scanner (LI-COR).

Ectopic SpeB Expression

Human codon-optimized open reading frames encoding the mature form of SpeB and a catalytically inactive SpeB-C192S derivative were purchased from Genscript as N-myc-tagged expression constructs in pcDNA3.1(+). HEp2 cells grown on glass coverslips were transfected with myc-SpeB or myc-SpeB-C192S. At 6 hr posttransfection, cells were fixed in 4% PFA and permeabilized with 0.1% Triton X-100. SpeB was detected with anti-myc antisera; NDP52, p62, and NBR1 were detected with mouse monoclonal antibodies. Nuclei were stained with DAPI. Confocal z stacks were acquired using inverted confocal microscope Zeiss LSM 710 (Plan-Apochromat 63×/1.4 Oil DIC objective) operated by ZEN2009 acquisition software. Intracellular structures positive for respective antigens were quantified using image segmentation and particle analysis in ImageJ 1.46a (Wayne Rasband, NIH, USA).

Transmission Electron Microscopy

HEp-2 cells cultured on glass coverslips were infected with GAS strains as described above at a multiplicity of infection equal to 8. At 6 hr postinfection, the cells were fixed in glutaraldehyde, postfixed in osmium tetroxide, dehydrated in ethanol series, flat-embedded in EPON resin, and polymerized at 60°C. Thin sections were cut on Leica EM UC6 microtome and contrasted with uranyl acetate and lead citrate. Images were acquired on Jeol EM 1011 electron microscope operating at 100 kV.

Statistics

For statistical analysis, the mean ±SEM for three independent experiments is shown in figures unless otherwise stated, and p values were calculated using a one-tailed Student's t test. A p value of less than 0.05 was determined to be statistically significant.

ACCESSION NUMBERS

The Illumina genome sequence data of M1T1^{5448} and M1T1^{5448} ΔspeB were deposited into the ENA (http://www.ebi.ac.uk/ena/) under the accession numbers ERS351322 and ERS351323, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.11.003.

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